

Enhancer competition between *H19* and *Igf2* does not mediate their imprinting

JENNIFER V. SCHMIDT, JOHN M. LEVORSE, AND SHIRLEY M. TILGHMAN*

Department of Molecular Biology and The Howard Hughes Medical Institute, Princeton University, Princeton, NJ 08544

Contributed by Shirley M. Tilghman, June 21, 1999

ABSTRACT The linked *H19* and *Igf2* genes on mouse distal chromosome 7 are subject to genomic imprinting. Competition between the promoters of the genes for transcription from shared enhancers has been proposed as an explanation for the coordinate expression and reciprocal imprinting of these two genes. To test this model, we have used Cre-loxP technology to generate in mice a conditional deletion of the *H19* promoter and structural gene that leaves no transcription unit in the locus. Contrary to the prediction of enhancer competition we find that transcriptional activity from the *H19* promoter is not required for the imprinted silencing of the *Igf2* gene.

A small number of genes in the mammalian genome are subject to genomic imprinting, the parent-of-origin-specific expression of the two alleles of a gene. To date, nearly 30 imprinted genes have been identified in human and mouse, the majority of which are located in close proximity to at least one other imprinted gene (1, 2). This clustering has raised the possibility that imprinting may be regulated by regional signals that act over a chromosomal domain. One imprinted region that has been studied intensively lies at the distal end of mouse chromosome 7 and the corresponding region of human chromosome 11p15.5. This region spans approximately 1 Mb and includes the paternally expressed *Igf2* gene and the maternally expressed *H19* gene (1).

A functional link between the imprinting of *H19*, which encodes an untranslated RNA, and *Igf2*, which encodes a fetal growth factor, was suggested by the observation that the genes are coexpressed throughout development in tissues of endodermal and mesodermal origin (3, 4). We have proposed previously that the coexpression and reciprocal imprinting of *Igf2* and *H19* could result from the promoters of the *H19* and *Igf2* genes competing for enhancers located downstream of the *H19* gene (5). On the maternal chromosome the *H19* gene would monopolize the enhancers by virtue of its proximity and/or greater promoter strength, whereas on the paternal chromosome, allele-specific methylation of *H19* would silence the gene and allow *Igf2* access to the enhancers. One prediction of this model was verified when two endodermal enhancers that lie 3' of *H19* were shown to be required for transcription of both genes (6). A second prediction, that silencing of *H19* by DNA methylation is required for *Igf2* expression, was tested in homozygous mutant mice lacking the maintenance DNA methyltransferase (7). The activation of paternal *H19* transcription that results from the loss of DNA methylation was accompanied by the silencing of *Igf2* in cis, a finding consistent with enhancer competition.

The third prediction of this model is a requirement for *H19* transcription on the maternal chromosome to maintain the silence of the maternal *Igf2* allele. This prediction has been tested with two mutations that deleted the *H19* gene and its

promoter. The first mutation (*H19*^{Δ13}) replaced 13 kb of the locus, encompassing the *H19* transcription unit and its flank, with a neomycin resistance gene (Neo) (8). The resulting mice exhibited extensive loss of imprinting at *Igf2*, expressing the maternal *Igf2* allele at between 30% and 100% of the paternal levels in a variety of tissues. A smaller deletion that replaced just 3 kb of the *H19* gene body and promoter with a different Neo gene cassette (*H19*^{Δ3}) reported a modest relaxation of the *Igf2* imprint in skeletal muscle, with maternal *Igf2* mRNA levels that were 25% of the paternal level (9). Although both mutations eliminated *H19* transcription, they left another transcription unit at the locus, the Neo cassette, a potential competitor for *Igf2* transcription. Thus, the differences in their phenotypes could have resulted from the transcriptional properties of the selectable markers, rather than the importance of the deleted DNA sequences.

The definitive test of enhancer competition requires a deletion of the *H19* promoter that leaves no transcription unit, i.e., no competitor, at the locus. We have generated this deletion by using Cre-loxP gene targeting and find that, contrary to the prediction of the enhancer competition model, the absence of transcription from the *H19* promoter has no effect on the imprinting of *Igf2* in liver and only a small effect in skeletal muscle. Our data lead us to propose a methylation-regulated chromatin boundary as an alternative mechanism for *Igf2* imprinting.

MATERIALS AND METHODS

Construction of the *H19* Targeting Vector. Two 129Sv/J genomic clones covering the *H19* gene and its flanks that have been described previously were used to generate the *H19*loxP targeting vector (10). The 5' homologous fragment is 5.5 kb and extends from a *Bgl*II site at -2 kb relative to the *H19* transcriptional start site to a *Sal*I site at +2.6 kb. The 3' homologous fragment is 7.5 kb and extends from the *Sal*I site to a *Bgl*II site at +10 kb. The 5' arm was modified by the insertion of a loxP site into the *Eco*RV site at -235 bp, and the 3' arm was modified by the insertion of the P_{gk}1-Neo cassette followed by a second loxP site into the *Sal*I site. The two modified clones were ligated into pBKSII to generate the targeting construct *H19*loxP, which was linearized for electroporation at a unique vector *Not*I site.

Generation of Conditionally Targeted and Null Alleles at the *H19* Locus. The *H19*loxP targeting vector was electroporated into E14 embryonic stem (ES) cells followed by selection in 250 μg/ml G418. Resistant colonies were screened by Southern blotting with a 500-bp, PCR-generated *H19* fragment from -2.2 kb to -2.7 kb (5' probe) and verified at the 3' end with a 1-kb *Bgl*III-*Eco*RI fragment (3' probe). Correctly targeted clones were obtained at a frequency of 1 in 40 G418-selected clones. *H19*loxP ES cell clones were transiently trans-

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Abbreviation: ES, embryonic stem.

*To whom reprint requests should be addressed at: Department of Molecular Biology, Washington Road, LTL128, Princeton, NJ 08544. E-mail: stilghman@molbio.princeton.edu.

fectured with the Cre expression vector pBS185 (11), replicated, and screened for survival in G418. Clones that died in G418 medium were analyzed by Southern blotting with the 5' probe to verify excision of *H19*-Neo.

RNA and DNA Analysis. All animals were analyzed between 3 and 5 days after birth. Liver and tongue (skeletal muscle) RNA was extracted with Trizol (GIBCO/BRL) for RNase protection analysis or with LiCl for reverse transcription-PCR. RNase protection analysis was performed with the RPAII kit (Ambion) by using 5 μ g of total RNA for *H19* assays and 10 μ g of total RNA for *Igf2* assays. RNase protection probes have been described previously and include a 120-bp *SmaI*-*Bam*HI fragment of the first exon of the mouse *H19* gene (12), a 350-bp, PCR-generated fragment of the mouse *Igf2* gene 3' untranslated region [*Igf2* allele-specific (8)] and a 270-bp *Xho*II-*Dra*I fragment of the mouse rpl32-4A gene (13). RNase protection gels were exposed to x-ray film and quantitated by use of a Molecular Dynamics PhosphorImager.

Southern blotting was performed with 5 μ g of digested mouse genomic DNA and analyzed by electrophoresis in 0.8% agarose gels. DNA was transferred to Hybond N+ membranes and hybridized in 5 \times SSPE (0.18 M NaCl/10 mM phosphate, pH 7.4/1 mM EDTA) with random-primed probes. The blots were washed twice at 2 \times SSPE/0.1% SDS, once at 1 \times SSPE/0.1% SDS and once at 0.1 \times SSPE/0.1% SDS. Radio-labeled probes for methylation analysis were a 3.8-kb *Eco*RI fragment comprising from -40 to -3,800 bp of the *H19* 5' flanking region, and a 717-bp fragment covering the *Igf2* DMR1 region.

RESULTS AND DISCUSSION

Generation of a Floxed Allele at the *H19* Locus. The RNA product of the *H19* gene has been shown to be dispensable for its own imprinting and that of *Igf2* (10). Transcription from the *H19* promoter, on the other hand, could be required in the germ line during the erasure and/or resetting of the epigenetic mark, as well as in the soma to regulate *Igf2* silencing. To specifically test the latter, we adopted a strategy that would allow us to delete the *H19* gene and promoter after the maternal chromosome had received its appropriate epigenetic modification during female gametogenesis. A gene-targeting vector was generated in which the *H19* gene was flanked with loxP sites (floxed) without altering the structural gene or its transcriptional control elements (*H19loxP* allele) (Fig. 1A). The lox site at the 5' end of the gene was integrated at -235 bp, a region that is not conserved between the human and mouse genes (14). The 3' lox site was integrated at a *Sal*I site 320 bp downstream of the polyadenylation signal of the gene. After electroporation into ES cells, G418-resistant clones were screened for the targeted allele by Southern blotting with a 5' probe that detects a 7-kb *Kpn*I fragment in wild-type ES cells and a 9-kb fragment in correctly targeted ES cells (Fig. 1). Targeted clones also were checked at the 3' end, where an 11-kb *Eco*RI fragment in wild-type ES cells is converted to a 13-kb fragment in targeted clones (Fig. 1A and data not shown). Correctly targeted *H19loxP* clones were microinjected into C57BL/6 blastocysts, and chimeras were bred to C57BL/6 mice to assay for germ-line transmission. Two independent clones that gave germ-line transmission were studied further.

Analysis of *H19loxP* Mice. To verify that the insertion of the loxP sites and Neo cassette did not disrupt expression of the *H19* gene, *H19* RNA in neonatal liver was quantitated by RNase protection analysis with rpl32 as an internal standard (Fig. 2). We use the designations *H19loxP*^{Mat} and *H19loxP*^{Pat} to indicate animals inheriting the *H19loxP* allele maternally and paternally, respectively. *H19loxP*^{Mat} animals exhibited a reduction in total *H19* mRNA levels in both liver and skeletal muscle to approximately 60% of that seen in wild-type litter-

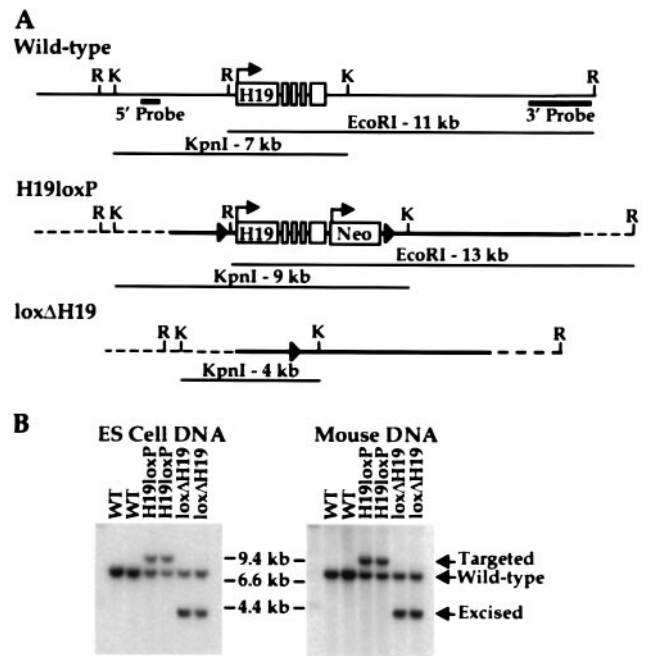


Fig. 1. Conditional targeting of the *H19* locus. (A) The structures of the wild-type, targeted, and excised *H19* loci are shown along with diagnostic restriction fragments. The solid arrowheads represent the loxP sites, the solid lines represent DNA contained within the targeting construct, and the dashed lines represent DNA outside the targeting construct. The two probes used for Southern analysis of ES cell clones and mouse DNA are shown and are located outside of the region contained within the targeting construct. Restriction sites are abbreviated as *Eco*RI (R) and *Kpn*I (K). (B) *Kpn*I-digested ES cell and mouse genomic DNA was hybridized to the 5' probe, which detects a 7-kb fragment in wild-type (WT) *H19* alleles, a 9-kb fragment in the floxed (*H19loxP*) allele, and a 4-kb fragment in the deletion allele (*loxΔH19*).

mates, whereas the expression of wild-type maternal *H19* RNA in *H19loxP*^{Pat} mice was unaffected (Fig. 2).

The decrease in *H19* RNA likely is due to a reduction in transcription rather than to a change in RNA stability because the loxP sites lie outside the transcription unit. It is possible that one or both of the loxP sites disrupt a hitherto unmapped regulatory element. More likely, the reduction in *H19* expression is caused by the presence of the Neo gene that is integrated just downstream of *H19* (Fig. 1A). Interference by the *Pgk*I promoter, either directly or through competition for

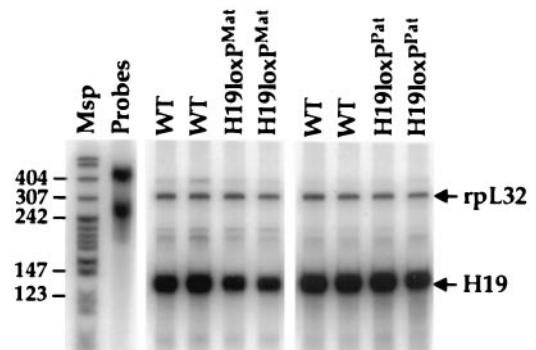


Fig. 2. *H19* RNA expression in *H19loxP* heterozygotes. A quantitative RNase protection assay was used to determine the levels of *H19* RNA in livers of wild-type (WT) mice and mice carrying the *H19loxP*^{Mat} and *H19loxP*^{Pat} alleles. The upper band indicates the level of expression of an internal control RNA (rpl32). The Msp lane contains *Msp*I-digested pBR322 as a size standard, and the Probes lane contains undigested probes.

enhancers, could result in a decrease in total *H19* RNA levels. We have observed a similar effect of the Neo cassette in two targeted alleles at the *Ednrb* locus (M. K. Shin and S.M.T., unpublished results), suggesting that the effect is unrelated to imprinting.

Interestingly, the *H19loxP^{Mat}* animals showed no relaxation of *Igf2* imprinting in liver and skeletal muscle despite the decrease in *H19* transcription (data not shown). Taken alone this observation does not argue for or against enhancer competition, because the combined transcription of *H19* and Neo still could be sufficient to restrict *Igf2* transcription.

Conditional Deletion of the *H19* Gene in the Maternal Germ Line. To examine the consequences of deleting transcription at the *H19* locus in the soma, we took advantage of a transgene that expresses Cre recombinase in growing oocytes under the direction of the oocyte-specific Zp3 promoter (15). By this stage of oogenesis it is thought that the female *H19* gametic mark has been established based on the erasure of *H19* methylation and its biallelic expression (16, 17). Animals carrying the *H19loxP* allele were crossed to Zp3Cre transgenics, and compound heterozygous females were bred to *Mus castaneus* males to provide polymorphisms for allelic RNA analysis. When the progeny of this cross were analyzed, $\approx 50\%$ of the animals carried the *H19* deletion, referred to as *lox Δ H19^{Oocyte}*. Southern blotting demonstrated the loss of the 9-kb targeted *KpnI* fragment and the appearance of a new 4-kb band, diagnostic of Cre-mediated excision of *H19* and Neo (Fig. 1). The *lox Δ H19^{Oocyte}* animals showed a complete absence of the targeted *H19* DNA in both liver and skeletal muscle, indicating that excision occurred before fertilization (data not shown). This is consistent with the previous report demonstrating the efficacy of Zp3Cre in the female germ line (15).

***H19* and *Igf2* Expression in *lox Δ H19^{Oocyte}* Heterozygotes.** Animals in which the *H19* transcription unit was deleted during oogenesis displayed no *H19* RNA in liver and skeletal muscle, indicating that the deletion had no effect on the imprinting of the paternal *H19* gene (data not shown). An allele-specific RNase protection assay for *Igf2* mRNA was used to investigate its imprinting status after excision of the *H19* gene (Fig. 3A). In contrast to the prediction of the enhancer competition model, *lox Δ H19^{Oocyte}* animals showed no loss of *Igf2* imprinting in liver. This finding was confirmed by quantitative RNase protection assays, which revealed no difference between *Igf2* mRNA levels in *lox Δ H19^{Oocyte}* and wild-type littermates (data not shown). The maintenance of *Igf2* imprinting in the livers of *lox Δ H19^{Oocyte}* animals provides convincing evidence that enhancer competition between the *H19* and *Igf2* promoters does not underlie the silencing of the maternal *Igf2* allele. In contrast to the finding in liver, *lox Δ H19^{Oocyte}* pups exhibited a small amount of relaxation of *Igf2* imprinting in skeletal muscle (Fig. 3A). The degree of relaxation is significantly less than the 100% relaxation of *Igf2* imprinting seen in the *H19^{Δ13}* maternal heterozygotes and somewhat less than the 25% relaxation reported for *H19^{Δ3}* heterozygotes (8, 9).

The tissue-specific relaxation of *Igf2* imprinting in skeletal muscle is difficult to explain. Of the four targeted mutations that have been generated at the *H19* locus, the only one that displays no relaxation of *Igf2* imprinting in skeletal muscle is the *H19^{Luc}* mutation (10). This mutation alone preserves the endogenous *H19* promoter, and, consequently, we cannot rule out a minor role for the *H19* promoter in the maintenance of the *Igf2* imprint in skeletal muscle. Alternatively, the sensitivity of *Igf2* imprinting in skeletal muscle to any perturbation in the *H19* locus could be related to the nature or location of the tissue-specific transcriptional regulatory elements that act in mesoderm to regulate *Igf2* and *H19*, about which very little is known.

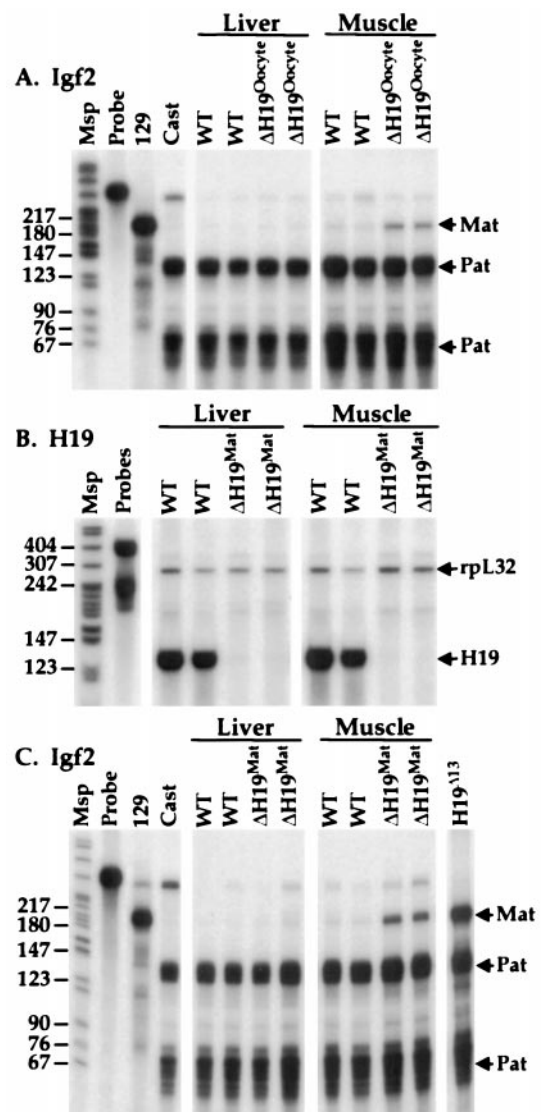


FIG. 3. *H19* and *Igf2* expression in *lox Δ H19^{Oocyte}* and *lox Δ H19^{Mat}* mice. (A) The imprinting status of the *Igf2* gene in liver and skeletal muscle of wild-type (WT) mice and mice carrying the *lox Δ H19^{Oocyte}* deletion were examined by an allele-specific RNase protection assay. The Msp lane contains *MspI*-digested pBR322 as a size standard, and the Probe lane contains undigested probes. The 129 and Cast lanes contain liver RNA samples from the parental 129 and *M. Castaneus* mice. Mat and Pat refer to the parental origin of the RNase protection bands. (B) *H19* RNA was analyzed by an RNase protection assay in wild-type (WT) mice and mice carrying the *lox Δ H19^{Mat}* allele. (C) The imprinting status of the *Igf2* gene in liver and skeletal muscle of wild-type (WT) mice and mice carrying the *lox Δ H19^{Mat}* deletion was examined by an allele-specific RNase protection assay as in A. The *lox Δ H19^{Δ13}* lane contains liver RNA from a mouse with maternal inheritance of the *H19^{Δ13}* deletion, where the maternal expression of *Igf2* mRNA has been determined previously to be approximately 30% of paternal expression (8).

Germ-Line Inheritance of *lox Δ H19^{Mat}*. In light of the modest consequence of excising the *H19* transcription unit during oogenesis, we went on to ask whether transcription from the *H19* promoter is required during gametogenesis to set the sex-specific epigenetic states of the parental chromosomes. *H19loxP*-targeted ES cells were transiently transfected with the plasmid pBS185, which expresses the Cre recombinase (11), and clones were replica-plated into G418. G418-sensitive clones were analyzed by Southern blotting by using the 5' probe to detect the loss of the 9-kb targeted *KpnI* fragment and the appearance of a new 4-kb band (Fig. 1). Two independent

clones that gave germ-line transmission for the deletion, referred to as $\text{lox}\Delta H19$, were studied.

The heterozygous female offspring of male chimeras were crossed to *M. castaneus* males, and the allelic expression of *H19* and *Igf2* was assessed in their progeny. This cross tests whether the $\text{lox}\Delta H19$ allele is capable of switching from a paternal epigenotype to a maternal one during oogenesis in the absence of the *H19* gene. As expected, no *H19* RNA was detected in $\text{lox}\Delta H19^{\text{Mat}}$ offspring (Fig. 3B). Furthermore, as was noted with $\text{lox}\Delta H19^{\text{Oocyte}}$ progeny, *Igf2* mRNA imprinting was maintained in liver, and minimal relaxation of imprinting was detected in skeletal muscle (Fig. 3C). Quantitative RNase protection demonstrated a 17% increase in total skeletal muscle *Igf2* mRNA, and this increase likely accounts for the fact that the $\text{lox}\Delta H19^{\text{Mat}}$ animals are approximately 5% larger than their wild-type littermates (data not shown). The similar behaviors of $\text{lox}\Delta H19^{\text{Oocyte}}$ and $\text{lox}\Delta H19^{\text{Mat}}$ animals argue that transcription from the *H19* promoter is not required for setting the maternal epigenetic mark.

Germ-Line Inheritance of $\text{lox}\Delta H19^{\text{Pat}}$. Offspring of male $\text{lox}\Delta H19$ heterozygotes were analyzed for their ability to maintain the paternal epigenetic mark in the absence of the *H19* gene. No differences between wild-type and $\text{lox}\Delta H19^{\text{Pat}}$ mutant littermates were observed in the levels of *H19* or *Igf2* expression or imprinting (Fig. 4). Additionally, heterozygous male offspring of $\text{lox}\Delta H19$ female heterozygotes were crossed to *Mus spretus* *H19-Igf2* congenic female mice. This cross tests the ability of the $\text{lox}\Delta H19$ allele to switch from a maternal to a paternal epigenotype. No alterations were found in the expression or imprinting of either *H19* or *Igf2* in the progeny of this cross. Animals homozygous for the $\text{lox}\Delta H19$ allele also were bred to *Mus spretus* *H19-Igf2* congenic mice, and their offspring were found to display phenotypes identical to those seen in the heterozygous crosses (data not shown).

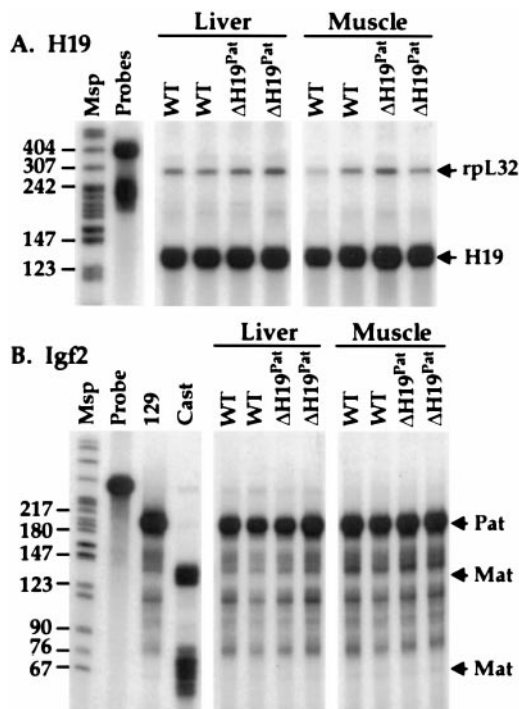


FIG. 4. *H19* and *Igf2* expression in $\text{lox}\Delta H19^{\text{Pat}}$ mice. (A) *H19* RNA was analyzed by an RNase protection assay in wild-type (WT) mice and mice carrying the $\text{lox}\Delta H19^{\text{Pat}}$ allele. Lane designations are as in Fig. 2. (B) The imprinting status of the *Igf2* gene in liver and skeletal muscle of WT mice and mice carrying the $\text{lox}\Delta H19^{\text{Pat}}$ deletion were examined by an allele-specific RNase protection assay. Lane designations are as in Fig. 3A.

Methylation Status of the *H19* and *Igf2* Genes in cis to $\text{lox}\Delta H19$. To assess whether the absence of transcription from the *H19* promoter had altered the ability of the chromosome to acquire the appropriate patterns of DNA methylation, we examined methylation of the *H19* and *Igf2* genes in $\text{lox}\Delta H19$ heterozygotes. The *H19* gene is heavily methylated on the paternal chromosome and largely unmethylated on the maternal chromosome. In particular, a 2-kb region between -2 and -4 kb is methylated exclusively on the paternal allele at almost all CpG residues (17, 18). This methylation is inherited from sperm, and maintained throughout embryogenesis, and thus is believed to represent the gametic mark for *H19*.

To assess the methylation status of this region in $\text{lox}\Delta H19^{\text{Mat}}$ and $\text{lox}\Delta H19^{\text{Pat}}$ animals, liver DNA was digested with *Sac*I, an enzyme that detects an *H19* polymorphism between 129 and *M. castaneus* mice, in the absence or presence of the methylation-sensitive restriction enzyme *Hpa*II or its methylation-insensitive isoschizomer *Msp*I. The maternally inherited *H19* gene was unmethylated in both wild-type and the $\text{lox}\Delta H19^{\text{Mat}}$ mice, as demonstrated by the nearly complete digestion of the 3.7-kb *Sac*I fragment (Fig. 5A). In $\text{lox}\Delta H19^{\text{Pat}}$ mice the 5' flanking region of the mutant gene was completely methylated, as shown by the maintenance of the 3.7-kb fragment in both wild-type and $\text{lox}\Delta H19^{\text{Pat}}$ animals. These results demonstrate that transcriptional activity from the *H19* promoter is not required to establish or maintain parent-of-origin-specific methylation patterns.

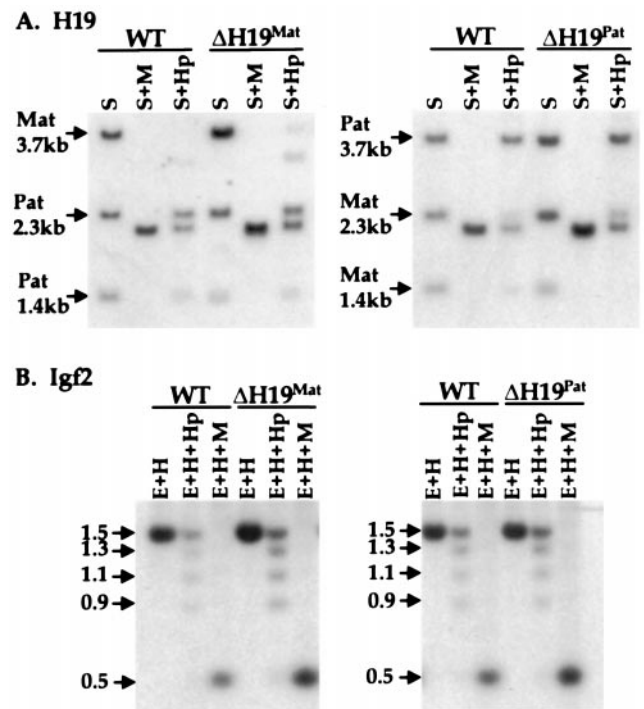


FIG. 5. Methylation of *H19* and *Igf2* in $\text{lox}\Delta H19$ heterozygotes. (A) Methylation analysis of the *H19* 5' flanking region. DNA from wild-type (WT) mice and mice carrying $\text{lox}\Delta H19^{\text{Mat}}$ (Left) or $\text{lox}\Delta H19^{\text{Pat}}$ (Right) alleles was digested with *Sac*I (S) and *Sac*I plus *Hpa*II (Hp) or *Msp*I (M). The blots were hybridized to a 3.8-kb *Eco*RI probe from the 5' flank of the *H19* gene. The partially methylated maternal *H19* DNA in $\text{lox}\Delta H19^{\text{Mat}}$ animals is more apparent than in wild-type animals because of loading differences. (B) Methylation analysis of the *Igf2* DMR1 region. Liver genomic DNAs from the same animals as in A were digested with *Eco*RI (E) and *Hind*III (H) or with *Eco*RI-*Hind*III plus *Hpa*II or *Msp*I. The blots were hybridized to a 717-bp probe from the DMR1 region of the *Igf2* 5' flank. In these animals, the 1.5-kb band represents the fully methylated paternal allele whereas the reduced methylation on the maternal allele gives a range of smaller digestion products.

The *Igf2* gene displays two regions of differential methylation, the DMR1 region located upstream of promoter P1 and the DMR2 region in the 3' part of the gene (19, 20). The allele-specific methylation of these regions is not established until after fertilization, and, unlike *H19*, it is the transcriptionally active allele of *Igf2* that is more highly methylated. Although this methylation does not qualify as a gametic mark, nor is it required for *Igf2* transcription (10), it does appear to correlate with expression of *Igf2* (21).

To examine DMR1 methylation in *loxΔH19* mice, liver DNA was digested with *EcoRI* and *HindIII* in the presence or absence of *HpaII* or *MspI* and analyzed by Southern blotting with a DMR1 probe (Fig. 5B). In wild-type mice, the methylated paternal allele appears as a 1.5-kb fragment whereas partial methylation of several maternal *HpaII* sites leads to multiple digestion products ranging from 500 bp to 1.3 kb. DNA samples from *loxΔH19^{Mat}* and *loxΔH19^{Pat}* animals show a pattern identical to those of their wild-type littermates.

A Boundary Model for the Imprinting of *H19* and *Igf2*. Our data have demonstrated conclusively that transcription from the *H19* promoter is not required for the silencing of the maternal *Igf2* gene in cis. This rules out strict enhancer competition between the *H19* and *Igf2* gene promoters to explain the coexpression and reciprocal imprinting of these two genes. At best, the *H19* promoter may play a minor role in silencing the maternal *Igf2* allele in skeletal muscle. When considered together with the recent findings of Thorvaldson *et al.* (22) our results argue that the sequences that regulate *Igf2* silencing must lie exclusively upstream of the *H19* transcription unit. Thorvaldson *et al.* (22) generated a 1.6-kb deletion within the candidate gametic mark upstream of the *H19* gene. Mice carrying this deletion exhibit a complete loss of imprinting of both *H19* and *Igf2*, with both genes coexpressed at reduced levels on both parental chromosomes. Those authors argued that their findings could be reconciled with enhancer competition if the epigenetic region was required for high-level *H19* transcription on the maternal chromosome. This interpretation cannot be sustained in light of our results.

What is the mechanism by which the flank of the *H19* gene silences the *Igf2* and *Ins2* genes 90 kb away? We currently favor the idea that the region functions as a chromatin boundary or insulator element on the unmethylated maternal chromosome (23). Boundary elements are defined as cis-acting DNA sequences that delineate specialized gene expression domains in chromatin (24, 25). When placed on either side of a gene and its regulatory elements, they insulate the gene from regulatory elements of neighboring genes. In other circumstances, boundary elements are used to segregate regulatory elements that act on the same gene in different cells into separate functional domains (26). At the *H19* gene, we suggest that the epigenetic control region forms a boundary on the maternal chromosome that blocks the access of *Igf2* to the downstream enhancers, resulting in the exclusive expression of the *H19* gene. On the paternal chromosome the boundary cannot form in the presence of DNA methylation, and, therefore, *Igf2* has access to the enhancers. Transcription of *H19* is inhibited by spreading of the methylation from the epigenetic region into the promoter.

A boundary can rationalize much of the experimental data that have been generated to understand the mechanism of *Igf2* imprinting. The model explains the extensive relaxation of *Igf2* imprinting observed with the *H19^{Δ13}* deletion (8) and the smaller gametic mark deletion (22), both of which excised the putative boundary. In contrast, the *H19^{Δ3}* deletion and our *loxΔH19* deletion that left the boundary intact had no effect on *Igf2* imprinting in liver and only a modest effect in muscle [Ripoche *et al.* (9) and L. Dandolo, personal communication]. In an experiment in which the enhancers that normally lie downstream of the boundary were moved to a site upstream of the putative boundary, the direction of the maternal imprint was reversed, with *Igf2* expressed and *H19* silent (27). This

finding is precisely what would be expected if the epigenetic region functions as a boundary. Finally, the hypersensitivity of the 2-kb epigenetic region to nucleases on the maternal chromosome but not on the paternal chromosome is consistent with the nuclease sensitivity of well characterized boundaries in a variety of organisms (23, 28).

One way to reconcile a boundary model with a modified version of enhancer competition has been suggested by Geyer (24). She proposed that insulators could function as "decoy" or pseudo-promoters, blocking the interaction between downstream genes and their enhancers, in a directional manner, without producing functional transcripts. This explanation for insulator function has been suggested recently to explain the mechanism of action of the *scs* and *scs'* elements in *Drosophila*, the latter of which coincides with the promoter for a downstream gene (29). Because previous attempts to locate transcripts emanating from the *H19* 5' nuclease hypersensitive region between -2 and -4 kb using Northern analysis and RNase protection had been unsuccessful, we used the more sensitive reverse transcription-PCR to search for transcripts in the region. Using several PCR primer pairs spanning the region from -2 to -5.5 kb we detected very low level transcription across the *H19* 5' differentially methylated region and extending further upstream (data not shown). Semiquantitative reverse transcription-PCR analysis indicated that these transcripts are present at <0.1% of the level of *H19* RNA. These transcripts are difficult to characterize in detail because of their low abundance, but they raise the possibility that the upstream region, rather than the *H19* promoter, could be a target of enhancer competition with *Igf2*. On the other hand, transcription that does not yield stable transcripts has been detected across the human β -globin locus control region and may be a general property of cis-acting elements (30). The presence of these additional transcripts does not affect our conclusion that transcription from the *H19* promoter, as originally proposed in the enhancer competition model, is not required for *Igf2* imprinting.

The chromatin boundary model may be applicable to other imprinted loci. The intragenic epigenetic mark of the *Igf2^r* gene, which is methylated on the transcriptionally active maternal chromosome (31), could function on the paternal chromosome as a boundary for distal regulatory elements, resulting in the paternal-specific silencing of the gene. Likewise in the Prader Willi/Angelman syndromes gene cluster, the extensive maternal methylation at the 5' end of the *SNRPN* gene (32, 33) could function, in part, to inactivate a boundary for a neuronal enhancer required for expression of the distal Angelman candidate gene, *UBE3A*, which is imprinted only in brain (34, 35). It will be important in the future to consider the relative positions of epigenetic marks and regulatory elements to imprinted genes when proposing mechanisms of imprinting regulation.

We thank Dr. G. Martin for the *ZP3Cre* mice and members of the Tilghman laboratory for comments on the manuscript. This work was supported by a Jane Coffin Childs postdoctoral fellowship (J.V.S.) and a grant from National Institute of General Medical Sciences (S.M.T.). S.M.T. is an investigator of the Howard Hughes Medical Institute.

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